

REMARKS

Applicants gratefully acknowledge the helpful assistance that the Examiner provided in the January 5, 2004 telephone interview in clarifying the outstanding rejections and indicating the type of evidence necessary to overcome those rejections. Claims 18-20, 23-27, 31-34, 37-43, 46-50 and 52 are pending in this application.

The Invention

The claims pending in this application are directed to methods for obtaining genetically modified, human pluripotent hematopoietic stem cells (cells that are capable of giving rise to all hematopoietic lineages) via transformation with a vector comprising a heterologous gene. The claims are further directed to the identification of combinations of cytokines that allow these stem cells to proliferate without losing their pluripotency (i.e., without becoming more committed progenitors). This latter feature is necessary for obtaining such genetically modified stem cells, because only proliferating cells can be successfully transformed by many preferred methods. For this purpose applicants use CD34⁺Thy-

1⁺Lin⁻ cell populations, which comprise a small (approximately 5%) subpopulation of cells that are truly pluripotent.

Because of their ability to differentiate into all hematopoietic lineages, the genetically modified cells of this invention are most useful in a therapeutic setting (e.g., repopulation of the hematopoietic system of a patient whose hematopoietic system has been destroyed either by disease or by treatment of a disease). Such therapeutic benefit could not be achieved with more committed hematopoietic progenitors, or even with multipotent cells that can give rise to some, but not all, hematopoietic lineages. Thus, the ability to produce genetically modified, human hematopoietic stem cells which remain pluripotent represents a medically important breakthrough.

Drawings

Formal drawings that have been corrected in accordance with the requirements set forth in Paper No. 24 (dated December 31, 2003), accompany this response.

Claim amendments

Applicants have amended claim 20 and claim 40 to add the phrase, "wherein said concentration range does not cause differentiation of the human pluripotent hematopoietic stem cells". Support for this amendment is found, e.g., at p.31, lines 15-19 of the original application. Thus, these amendments add no new matter.

Claim rejections

The Examiner has maintained the rejection of claims 18-20, 23-27, 31-34, 37-43 and 46-50 and rejected new claim 52 under 35 U.S.C. §103(a) as being "unpatentable" over a combination of numerous references. The primary references - H. Ku et al., Blood, 88, pp. 4124-31 (1996) ("Ku"); M. Kobayashi et al., Int. J. Hematol., 66, pp. 423-34 (1997) ("Kobayashi"); Y. Ohmizono et al., Leukemia, 11, pp. 524-30 (1997) ("Ohmizono"); and V. Ramsfjell et al., J. Immunol., 158, pp. 5169-77 (1997) ("Ramsfjell") - are cited by the Examiner as purportedly showing the proliferation of primitive human hematopoietic progenitor cells using combinations of cytokines falling within the scope of applicants' claimed invention. As

demonstrated below, none of those references, nor any other references cited by the Examiner, demonstrate that the cells that did proliferate in their studies were true pluripotent human hematopoietic cells, as required by the claims.

The Examiner cites Ku as teaching that TPO and SF individually, as well as in combination, supports formation of multilineage colonies. Ku is inapplicable for two reasons. First, Ku studied mouse hematopoietic progenitors, while the instant invention is limited to human pluripotent hematopoietic progenitors. While there are similarities between the hematopoietic systems of mice and men, there are also significant differences. Thus, one cannot have a reasonable expectation that results observed for murine hematopoietic cells can be extrapolated to human hematopoietic cells.

More importantly, SF is a c-kit ligand and the claims pending in this application require, at a minimum, the presence of a mpl ligand (such as TPO) and a flt3 ligand (FL). Ku's experiments utilizing TPO and SF do not meet this minimum requirement. In fact, Ku does not utilize a cytokine combination comprising an mpl ligand and a flt3 ligand in any of the disclosed experiments. Thus, even if Ku did demonstrate

proliferation of pluripotent cells in the presence of SF and TPO, which it did not, it would still not render obvious the present invention. Thus, Ku's combination does not render the applicant's combination obvious.

In the experiment cited by the Examiner (Ku, Table 1), what the authors term "primitive progenitors" were treated with TPO and SF in the presence of EPO. The cells were grown for 14 days and the resulting colonies analyzed. Assuming there were any pluripotent cells in the starting population (and there is no proof of that), the resulting treatment caused differentiation into various "multilineage colonies", as well as into identifiable megakaryocytes ("Meg" in Table 1). According to Figure 2 in Ku, this "multilineage colony" was not a colony of cells each of which had multipotent or pluripotent potential, but rather, a mixture of differentiated cells (see Figure 2B which shows that a GEMM colony contained megakaryocyte, neutrophils and macrophages). This result is completely opposite to that of the present invention and therefore teaches away from it. The goal of the present invention is to identify conditions that allow proliferation of

the pluripotent stem cells without differentiation to more committed progenitors or to differentiated hematopoietic cells.

In the instant application, CD34⁺Thy-1⁻Lin⁻ cells were grown in the presence of various mixtures of cytokines in an attempt to identify conditions that caused proliferation of the pluripotent stem cells in that population. The dividing (PKH^{lo}) CD34^{hi} cells were isolated and incubated with a murine stromal cell line in a CAFC assay to demonstrate that they retained their full pluripotent potential¹ (See Example 4). This is very different from the experiments of Ku which never subjected its "primitive progenitor" cells to a full complement of differentiation-inducing cytokines (or growth on stromal cells), yet observed differentiation in the presence of only TPO and SF.

The Examiner also cites Kobayashi, Ohmizono and Ramsfjell as teaching that applicants' claimed combination of

¹ The murine stromal cell line produces all of the cytokines necessary to cause hematopoietic progenitor cells to differentiate. Applicants also analyzed non-dividing progenitor cells (PKH^{lo}CD34^{hi}) in the CAFC assay after growth in the presence of various cytokine combinations. That non-dividing subpopulation is representative of the starting CD34⁺Thy-1⁻Lin⁻ population and therefore contained pluripotent stem cells, as borne out by the assay results (see Table 2).

cytokines support the formation of multilineage colony formation. While the Examiner is correct that these references were related to studying multilineage cells, applicant's invention is patentably distinct from these references because it identifies cytokines which maintain the proliferative state of the pluripotent stem cell. All pluripotent cells are multilineage, but not all multilineage cells are pluripotent, with most being restricted to a narrower range of lineages to which they can give rise.

As defined on page 1, lines 6-11, of the present application, the hematopoietic stem cell of the invention is a pluripotent cell, which is capable of differentiating into any cell of the hematopoietic system. Applicants have attached as Exhibits A and B hereto, schematics of hematopoietic cell differentiation and lineages. Each of these documents contains a figure depicting the relationship and difference between a pluripotent cell and a multipotent cell (e.g., CLP or "lymphoid stem cell" and CMP or "myeloid stem cell"). These figures demonstrate schematically that the pluripotent hematopoietic stem cell gives rise to the multipotent cell and that each type

of multipotent cell can only give rise to certain subsets of mature blood cells.

None of Kobayashi, Ohmizono or Ramsfjell demonstrates that it has achieved proliferation of, or even possession of a true pluripotent cell. This is because none of those references ever test cells in an assay that can identify a pluripotent cell. In order to accurately assess whether a particular cytokine combination can support proliferation of pluripotent cells, one must distinguish between the proliferation of the non-pluripotent (e.g., multipotent and further differentiated) cells, the proliferation of pluripotent cells, and the proliferation and differentiation of pluripotent cells into more committed progenitors in the starting population. Assays such as the CAFC and most importantly, long-term engrafting potential studied in vivo are accepted assays for determining if a cell is truly pluripotent (see application, page 8, line 17 - page 9, line 9).

If anything, the assays employed by the cited references suggest that they were unsuccessful in obtaining a proliferating pluripotent cell.

"However, we could not determine whether the population recruited by Tpo also had lymphoid potential." (Ramsfjell, page 5176, left column, line 53-55).

* * * *

"Both FL and SF produced predominantly GM colony-forming cells in synergy with TPO" (Kobayashi, page 427, right column).

* * * *

"Our data demonstrate that it is possible to expand human CB-derived committed progenitors in vitro using SCF or FL with several other cytokines including TPO..." (emphasis added, Ohmizono, p. 524, abstract).

Moreover, neither Ohmizono nor Kobayashi ever even assayed for the ability of their proliferating cells to differentiate into cells of the lymphoid line.

In contrast, applicants have used CAFC assays (Example 4 and Table 3) and in vivo engraftment assays in SCID-hu mice (Example 5 and Table 4) to confirm that growth in the presence of the cytokines recited in the claims cause proliferation of pluripotent cells and retention of their pluripotency.

Moreover, since none of the cited references use the Thy-1+ marker employed by applicants to further purify their CD34+ cells, one would expect their starting frequency of true

pluripotent cells to be lower than the 1/20 previously shown for the CD34+Thy-1+Lin- population (see p.9, lines 2-6). Thus, as the experiments reported in those references typically started with between 1 and 100 cells, there exists the real possibility that none had even a single pluripotent cell to begin with.

The experiments in the references cited that show an increase in the number of cells from a starting population of CD34+ cells do not prove that there is a proliferation of the true pluripotent cells. This is because none of the experiments show that individually picked cells from the proliferating colonies can differentiate into all hematopoietic lineages. The observed proliferation and lineage-restricted differentiation could be attributable to selective proliferation and differentiation of more committed progenitors in the population or the absence of true pluripotent cells from the starting cultures.

Accordingly, none of these cited references teach or suggest, alone or in combination with any other references, cytokine combinations capable of causing proliferation of pluripotent cells without loss of pluripotency.

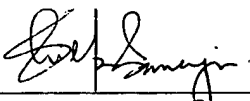
The Examiner also cites Kobayashi and Ohmizono for the teaching that IL-3 is required for the expansion of hematopoietic cells. As discussed above, neither of these references teach that IL-3, in combination with other cytokines, cause expansion of hematopoietic stem cells without causing differentiation. Rather, each of these references addresses the expansion of committed progenitors, and not pluripotent stem cells. To clarify that point further, applicants have amended claim 20 and 40 to indicate that the recited IL-3 concentrations cause proliferation of pluripotent hematopoietic stem cells without causing differentiation.

The Examiner has further cited Murray et al. (U.S. Patent 5,665,557), Nakahata (U.S. Patent 5,861,315), Hoffman et al. (U.S. Patent 5,744,361), Fei et al. (U.S. Patent 5,635,387) or Davis et al. (U.S. Patent 5,599,703) as references that, in view of several secondary references, render the claims obvious. None of these references, alone or in combination with any of the secondary references cited (Ku, Kobayashi, Ramsfjell, Ohmizono et al., Szilvassy et al., Escary et al., Bodine et al., Tushinski et al., Fletcher et al., Bello-Fernandez et al., Hatzfeld et al., or Hanenbergl et al. (Nature

Medicine) and Hanenberg et al. (IDS reference AR)) teach the cytokine combination of the present invention to support growth and not differentiation of pluripotent cells. Therefore, none of these references alone or in combination render the invention unpatentable.

For all the above reasons, applicants request that the Examiner withdraw the outstanding rejections and grant allowance to the pending claims.

Respectfully submitted,



James F. Haley, Jr. (Reg. No. 27,794)
Barbara A. Ruskin (Reg. No. 39,350)
Shilpi A. Banerjee (Reg. No. 53,965)
Attorneys for Applicants

FISH & NEAVE
Customer No. 1473
1251 Avenue of the Americas
New York, New York 10020-1105
Tel.: (212) 596-9000
Fax: (212) 596-9090

STEM AND PROGENITOR CELLS: Origins, Phenotypes, Lineage Commitments, and Transdifferentiations

Irving L. Weissman

*B257 Beckman Center, Stanford University School of Medicine Stanford,
California 94305-5323; e-mail: Irv@stanford.edu*

David J. Anderson

*Division of Biology, 216-76, California Institute of Technology Pasadena,
California 91125; e-mail: Wuwei@caltech.edu*

Fred Gage

*The Salk Institute, Laboratory of Genetics 10010 N. Torrey Pines Road,
La Jolla, California 92037; e-mail: Gage@salk.edu*

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■ **Abstract** Multipotent stem cells are clonal cells that self-renew as well as differentiate to regenerate adult tissues. Whereas stem cells and their fates are known by unique genetic marker studies, the fate and function of these cells are best studied by their prospective isolation. This review is about the properties of various highly purified tissue-specific multipotent stem cells and purified oligolineage progenitors. We contend that unless the stem or progenitor cells in question have been purified to near homogeneity, one cannot know whether their generation of expected (or unexpected) progeny is a property of a known cell type. It is interesting that in the hematopoietic system the only long-term self-renewing cells in the stem and progenitors pool are the hematopoietic stem cells. This fact is discussed in the context of normal and leukemic hematopoiesis.

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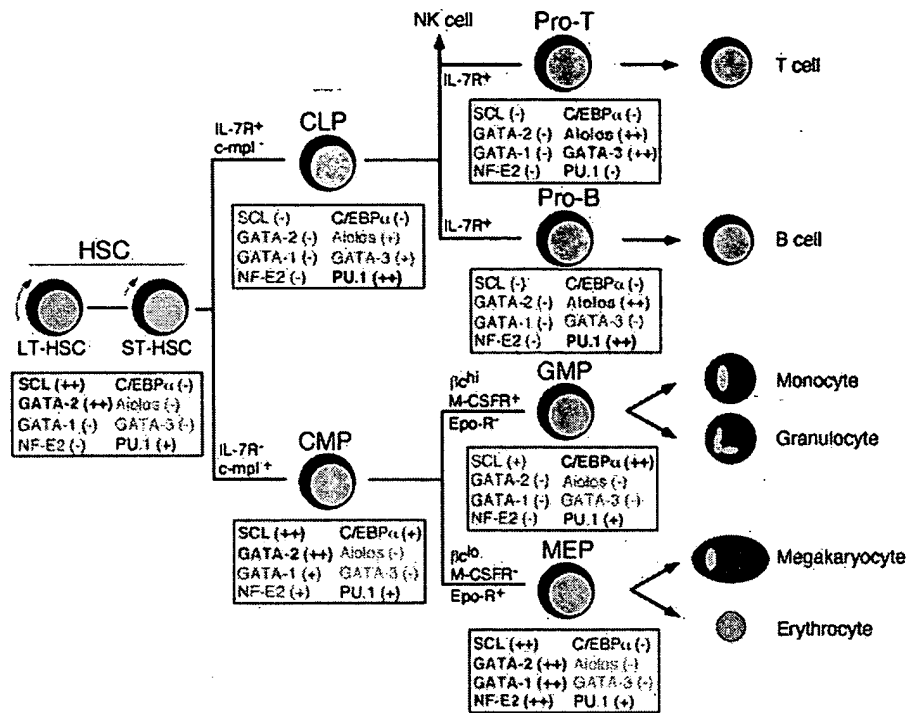


Figure 1 Proposed model of major hematopoietic maturation pathways from HSC. Long-term HSC (LT-HSC) give rise to short-term (ST) HSC. We propose that ST-HSC give rise to at least CLP, which can form all cells of the lymphoid lineage, and CMP, which can differentiate into either GMP or MEP, which form the cells of the granulocyte/macrophage or megakaryocyte/erythroid lineages, respectively. The three myeloid progenitor subsets should make up the vast majority of myeloid progenitor activity in steady-state bone marrow because the Sca-1⁻ c-kit⁺ fraction, which is composed of GMP, MEP, and CMP, and the Sca-1⁺ c-kit⁺ HSC fraction, were estimated to contain ~98% of myeloid colony-forming activity within the Lin- IL-7R α - fraction (data not shown). (From *Nature* 404: 193-97, 2000, with permission)

IMMUNOLOGY

THIRD EDITION

Ivan M Roitt MA DSc(Oxon) Hon MRCP(Lond) FRCPath FRS
Emeritus Professor of Immunology
Director of the Institute of Biomedical Science
University College London Medical School
London, UK

Jonathan Brostoff MA DM(Oxon) DSc FRCP FRCPath
Reader in Clinical Immunology
Department of Immunology
University College London Medical School
London, UK

David K. Male MA PhD
Senior Lecturer in Neuroimmunology
Department of Neuropathology
Institute of Psychiatry
London, UK

Editorial Consultant
Linda Gamlin BSc(Hons) MSc

M Mosby

St. Louis Baltimore Boston Chicago London Philadelphia Sydney Toronto

MYELOID CELLS

Myelopoiesis commences in the fetal liver of the human fetus at about 6 weeks of gestation. *In vitro* studies where colonies have been grown from individual stem cells, have shown that the first progenitor cell derived

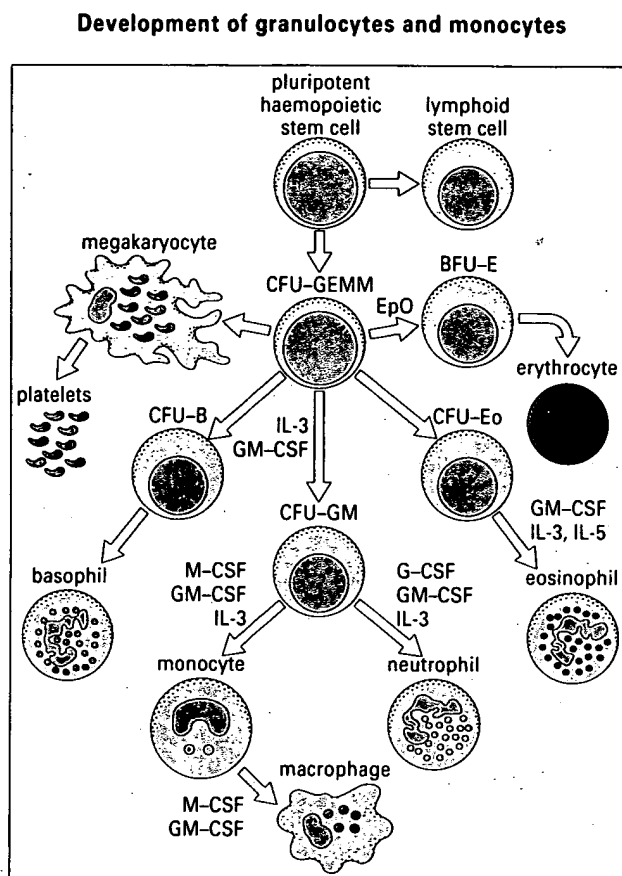
from the HSCs is the colony-forming unit (CFU), which can give rise to granulocytes, erythrocytes, monocytes and megakaryocytes (CFU-GEMM). Maturation of these cells occurs under the influence of colony-stimulating factors (CSFs) and several interleukins including IL-1, IL-3, IL-4, IL-5 and IL-6 (Fig. 11.2). These factors, which are important in the positive regulation of haemopoiesis, are derived mainly from stromal cells of the bone marrow, but are also produced by mature forms of differentiated myeloid and lymphoid. Other cytokines (e.g. TGF β) may down-regulate haemopoiesis.

GRANULOCYTE DEVELOPMENT

Induction of CFU-GM along the granulocyte pathway gives rise to distinct morphological stages of development. Myeloblasts develop into promyelocytes and myelocytes, and these mature further, to be released into the circulation as neutrophils, basophils or eosinophils. The one-way differentiation of cells from

Fig. 11.2 Pluripotent haemopoietic stem cells generate CFU-GEMMs which have the potential to give rise to all blood cells except lymphocytes. IL-3 and GM-CSF are required to induce this stem cell into one of five pathways (i.e. to give rise to megakaryocytes, erythrocytes via burst-forming units, basophils, neutrophils or eosinophils) and are also required during further differentiation of the granulocytes and monocytes. Eosinophil differentiation from CFU-Eo is promoted by IL-5. Neutrophils and monocytes are derived from the CFU-GM through the effects of G-CSF and M-CSF respectively. Both GM-CSF and M-CSF, and other cytokines (including IL-1, IL-4 and IL-6), promote the differentiation of monocytes into macrophages.

(B = basophil; BFU = burst-forming unit; CFU = colony-forming unit; CSF = colony-stimulating factor; E = erythrocyte; Eo = eosinophil; BFU = burst-forming unit; M = monocytes; MM = monocytes and megakaryocytes.)



Morphology and markers on developing granulocytes and monocytes

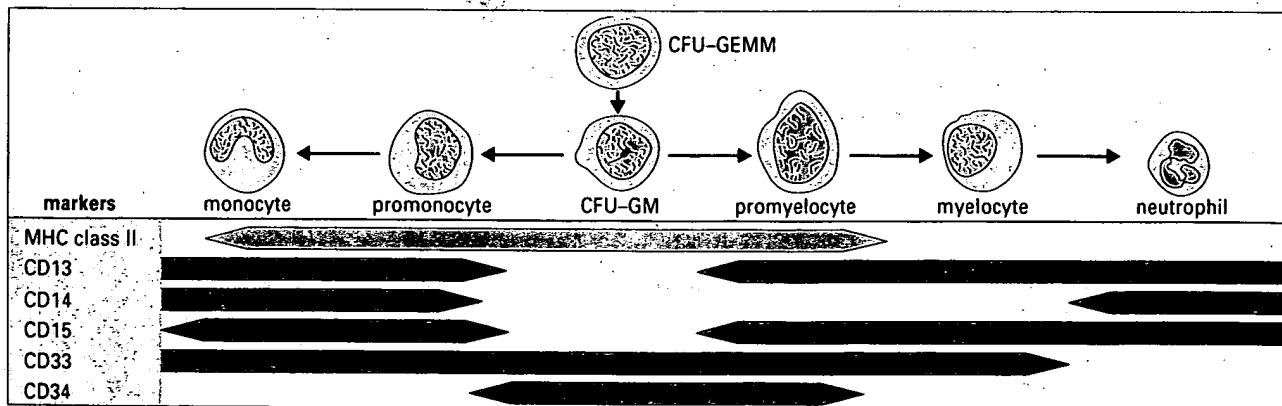


Fig. 11.3 Cells of the monocyte and granulocyte lineages develop from a common CFU-GM. Differentiation along either pathway results in loss of CD34. CD33 is maintained

on monocytes but is lost from mature neutrophils, as are MHC class II molecules. CD14 is expressed on monocytes but only weakly on some granulocytes (possibly activated).